

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant : Makoto Kikuchi et al.                      Art Unit : Unknown  
Serial No. : To be assigned                              Examiner : Unknown  
Filed : Herewith  
Title : COMPOSITION COMPRISING MIDKINE OR PLEIOTROPHIN PROTEIN  
AND METHOD OF INCREASING HEMATOPOIETIC CELLS

Commissioner for Patents  
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to examination, please amend the application as follows.

In the specification:

On page 1, delete the title ("NOVEL USE OF MK FAMILY AS HEMATOPOIETIC FACTOR") and insert in its place --"USE OF THE MK FAMILY AS HEMATOPOIETIC FACTORS".--

On page 1, after the title of the application, insert

--Cross Reference to Related Applications

This application is a divisional application of U.S. Application Serial No. 09/214,569, which is a National Phase Application, filed January 7, 1999, under 35 U.S.C §371, of International Application No. PCT/JP97/02401, filed July 10, 1997.--

Please replace the paragraph beginning on page 1, line 4, with the following rewritten paragraph:

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--This invention relates to a novel use of MK to promote proliferation and differentiation of hematopoietic stem cells and hematopoietic precursor cells in hematopoietic tissues, peripheral blood, or umbilical cord blood synergistically with other hematopoietic factors.--

Please replace the paragraph beginning on page 1, line 9, with the following rewritten paragraph:

--In blood, there exist various hemocytes having different shapes and functions, including erythrocytes, leukocytes, and platelets, which play important roles in maintaining homeostasis of the living body. These mature hemocytes have their own life-spans. For maintaining the hemocyte count at a constant level, hemocytes must be incessantly produced to make up for the number of hemocytes that is lost due to the expiration of their life-spans.--

Please replace the paragraph beginning on page 1, line 16, with the following rewritten paragraph:

--In the normal healthy individual, it is presumed that daily production of hemocytes reaches as much as  $2 \times 10^{11}$  erythrocytes,  $10^{11}$  leukocytes, and 1 to  $2 \times 10^{11}$  platelets. Hematopoietic stem cells play central roles in the system to produce such an enormous number of hemocytes over a long period without being exhausted. The cells have not only self-renewal capability but also multipotentiality to differentiate to various mature hemocytes including erythrocytes, granulocytes, platelets, and lymphocytes. Hematopoietic stem cells (multipotential stem cells) lose their self-renewal capability as they proliferate to become hematopoietic precursor cells (committed stem cells) destined to differentiate to the specific hemocytes. Hematopoietic precursor cells then differentiate to mature peripheral hemocytes.--

Please replace the paragraph beginning on page 2, line 5, with the following rewritten paragraph:

--It has been known that a number of cytokines regulate each step of the hematopoietic system to proliferate and differentiate hematopoietic stem cells to various mature hemocytes via hematopoietic precursor cells. At least twenty kinds of the cytokines participating in the hematopoietic system have been found at present (Masami Bessho: Igaku no Ayumi 180(13):

802-806, 1997). The genes for all have been cloned, allowing their production on a large scale by genetic engineering techniques. Stem cell factor (SCF) and flk-2 ligand are the most remarkable cytokines as factors acting on mainly hematopoietic stem cells at the early stage of hematopoiesis. SCF acts on the most undifferentiated hematopoietic stem cells. In either mice or humans, it remarkably promotes the formation of colonies of blast colony-forming unit (CFU-BL), colony-forming unit-mixed (CFU-Mix), burst forming unit-erythrocyte (BFU-e), colony-forming unit-granulocyte/macrophage (CFU-GM), eosinophil colony-forming unit (CFU-Eo), and colony-forming unit-megakaryocyte (CFU-Meg); showing a synergistic effect with various cytokines such as IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-11, G-CSF, GM-CSF, and EPO. It has been reported that SCF alone has weak colony-stimulating activity (Tsuji, K. et al., Blood 78: 1223, 1991; Shioharu, M. et al., Blood 81: 1453, 1993; Kubo, T. and Nakahata, T., Int. Hematol. 58: 153, 1993). Nevertheless, SCF is thought to be the most important cytokine for *in vitro* amplification of hematopoietic stem cells at present.--

Please replace the paragraph beginning on page 3, line 6, with the following rewritten paragraph:

--Some of these hematopoietic factors have been clinically applied. For example, erythropoietin (EPO), which promotes the production of erythrocytes, is used for treating renal anemia, and granulocyte colony-stimulating factor (G-CSF), which promotes the production of neutrophils is used for treating neutropenia caused by cancer chemotherapy. These contribute to improved quality of life of patients. Recently, the clinical application of thrombopoietin (TPO) for treating thrombocytopenia has been studied because it promotes the production of platelets.--

Please replace the paragraph beginning on page 3, line 15, with the following rewritten paragraph:

--On the one hand, since hematopoietic stem cells are capable of reconstituting all kinds of cells in the hematopoietic system, the transplantation of hematopoietic stem cells has been widely performed for hematopoietic tumors. Recently, the transplantation of peripheral blood stem cells has rapidly become prevalent, and gathered attention as the powerful fundamental therapy for the chemotherapy-sensitive malignant tumors including the hematopoietic organ

tumors. Furthermore, as a future prospect, the transplantation of hematopoietic stem cells is expected to be introduced to many cell therapy and gene therapy protocols. For that purpose, it is necessary to establish a method for amplifying hematopoietic stem cells *in vitro*. However, even now, human hematopoietic stem cells have been neither isolated nor clarified as to what extent they can repeat self-renewal.--

Please replace the paragraph beginning on page 4, line 23, with the following rewritten paragraph:

--MK was isolated as the product of a gene that is expressed at the early stage of the differentiation of mouse embryonic tumor cells by the induction of retinoic acid (Kadomatsu, K., et al., Biochem. Biophys. Res. Commun. 115: 1312-1318, 1988). PTN was found as a heparin-binding protein with neurite outgrowth capability in the newborn rat brain (Rauva, H., EMBO J. 8: 2933-2941, 1989). MK and PTN belong to a new class of heparin-binding growth factors, sharing a 45% homology (in amino acid sequence) to each other, and called the MK family. MK and PTN respectively exhibit characteristic expression patterns in the developmental process, indicating that they have important physiological activities for the implementation of differentiation.--

Please replace the paragraph beginning on page 5, line 8, with the following rewritten paragraph:

--Paying attention to such biological activities of the MK family, the present inventors studied their hematopoietic factor activities to proliferate and differentiate myeloid cells and peripheral blood stem cells of mammals. In general, at what stage of the proliferation/differentiation process of hematopoietic stem cells and hematopoietic precursor cells in myeloid hematopoietic factors participate and function can be studied by culturing a certain number of myelocytes in a semi-solid medium in the presence of these hematopoietic factors, selecting cells constituting colonies formed, and counting the number of colonies. In such colony formation methods, it has been proved by a number of direct or indirect methods that, a single hematopoietic precursor cell proliferates, divides, and matures, forming a single colony comprising many matured hemocytes. There are colony formation assay methods

specific for cells of each hematopoietic system including granulocyte/macrophage, erythroblasts, and megakaryocytes, and stimulators specific for each hematopoietic system are used.--

Please replace the paragraph beginning on page 5, line 25, with the following rewritten paragraph:

--Precursor cells of the granulocyte/macrophage system, CFU-GM, differentiate to precursor cells of neutrophil system, CFU-G, and precursor cells of monocytes, CFU-M. For that purpose, colony-simulating factors (CSF) specific to each precursor cell must be present. More specifically, GM-CSF is required for CFU-GM, G-CSF for CFU-G, and M-CSF for CFU-M. Some of these CSFs not only differentiate precursor cells to mature cells but also activate the function of matured hemocytes.--

Please replace the paragraph beginning on page 6, line 6, with the following rewritten paragraph:

--CFU-GM can be cultivated by either the soft agar method or the methylcellulose method using bone marrow nucleated cells. Since colonies formed by either method are constituted by a cell population of granulocytes and macrophages at various developmental stages, precursor cells one step further differentiated from hematopoietic stem cells are to be examined. Picking up and staining of these colonies revealed the presence of G colony consisting of granulocytes, M colony consisting of macrophages, and GM colony consisting of the mixture of both. In humans, colonies are rather small and classified into a group called a colony containing 40 cells or more and a group called a cluster with a lower accumulation of cells than a colony.--

Please replace the paragraph beginning on page 6, line 17, with the following rewritten paragraph:

--In order to examine whether MK has the activity causing myelocytes to proliferate, the proliferation of mouse myelocytes was assayed by the MTT method, resulting in enhancing the proliferation 1.6- to 2-fold in the system supplemented with MK at the concentrations of 5, 50,

500, and 5000 ng/ml as compared with the system without MK. A concentration-dependent elevation of the activity was observed in the range of 5 to 500 ng/ml MK.--

Please replace the paragraph beginning on page 6, line 24, with the following rewritten paragraph:

--In the colony assay for human peripheral blood mononuclear cells in the presence of various cytokines, as shown in Figure 1, colonies were not formed at all in the system without cytokines, but formed in the system supplemented with MK similarly as with GM-CSF and IL-3. The colony size tends to be larger in the system with MK added than in the systems with cytokines such as G-CSF, GM-CSF, and IL-3 added. These results indicate that MK alone has the activity to maintain the viability of human peripheral blood stem cells or hematopoietic precursor cells, or promote their proliferation. Furthermore, the combined use of MK with other cytokines such as M-CSF, G-CSF, GM-CSF, IL-3, and IL-6 synergistically promotes colony-forming capability. For example, the number of colonies increased 7 to 9-fold in the cases of combined use of MK with G-CSF, GM-CSF, or IL-3 as compared with those of the single use of MK, G-CSF, GM-CSF, or IL-3. Also, the combination of MK + G-CSF + IL-3 + IL-6 remarkably increased the number of colonies formed as compared with that of GM-CSF + IL-3 + IL-6, and the combination of MK + G-CSF + IL-6 significantly increased the number of colonies as compared with that of G-CSF + IL-6. In another experiment using a source of human peripheral blood mononuclear cells different from that used for the experiments described in Fig. 1, treatment with MK, GM-CSF, or IL-3 alone produced primarily GM colonies, while use of G-CSF alone produced primarily G colonies. Combinations of MK + G-CSF + GM-CSF, or of MK + G-CSF significantly increased the number of G colonies as compared with the use of G-CSF alone. That is, MK is considered to synergistically promote the proliferation, differentiation and maturation of CFU-GM of G-CSF, increasing the number of neutrophils in the peripheral blood.--

Please replace the paragraph beginning on page 7, line 25, with the following rewritten paragraph:

--When cells after 2-week liquid culture of human peripheral blood stem cells in the presence of various cytokines were examined by specific staining, there were observed, as shown in Figure 3, predominantly many granulocytes (neutrophils) in the system supplemented with MK, clearly indicating the action of MK on the proliferation of neutrophils. Especially, in the case of the combination of MK + G-CSF + GM-CSF + SCF + IL-3 + IL-6, there were observed an extremely remarkable promotion of the proliferation and differentiation of neutrophils.--

Please replace the paragraph beginning on page 8, line 6, with the following rewritten paragraph:

--Also, in the colony assay performed after the above-described liquid culture, the cell adherence to a culture dish increased in the system supplemented with MK as compared with that without MK, indicating that MK also promotes the proliferation of the interstitial cell system (stroma cell system). In the case of IL-6 alone, colonies formed were of macrophages, and in the case of MK + IL-6, half of colonies formed were of granulocytes. These results indicate the participation of MK in the promotion of proliferation and differentiation of granulocytes.--

Please replace the paragraph beginning on page 8, line 15, with the following rewritten paragraph:

--Whether such a remarkable promotion by MK of the production of neutrophils is displayed *in vivo* can be studied by administering MK to a mouse whose hematopoietic system has been damaged by administration of an anticancer drug, or exposure to radiation, and examining the recovery state of neutrophils. MK was administered to a mouse daily for 13 consecutive days, and on the 5<sup>th</sup> day after the initiation of administration, an anticancer drug, Cyclophosphamide (CY), was administered to the mouse. Examination of hemocytes in the blood collected from the mouse at appropriate intervals revealed a remarkable promotion of the recovery of the number of neutrophils as expected (Table 1).--

Please replace the paragraph spanning pages 9 and 10 with the following rewritten paragraph:

--Results of the colony assay for mouse spleen cells in the system of the M3434 medium supplemented with MK are shown in Figures 4 and 5. Figure 4 illustrates the number of CFU-GM colonies or CFU-G colonies. Although CFU-GM colonies or CFU-G colonies can be formed with the M3434 medium alone, the number of colonies generally increases in the system supplemented with the MK as compared with the M3434 medium alone. Especially, when MK is added at the concentration of 1 to 10 ng/ml, the colony number remarkably increased 2 to 3-fold on the 8<sup>th</sup> and 10<sup>th</sup> day of the assay. Figure 5 illustrates the number of colony-forming unit-mixed (CFU-Mix). CFU-Mix are multipotential stem cells at a slightly differentiated stage, having lower self-renewal capability than blast colony-forming units (CFU-BL) which are the most undifferentiated cells identifiable by the *in vitro* colony assay and are thought to contain cells capable of differentiating to erythrocytes, leukocytes, and platelets. In the system supplemented with MK, CFU-Mix colony significantly increased in number. That is, MK, at least by its combined use with IL-3, IL-6, SCF and EPO, is thought to significantly promote the proliferation and differentiation of hematopoietic stem cells or immature hematopoietic cells close to them. These activities are thought to be very useful for the proliferation of hematopoietic stem cells *in vitro* for the transplantation of bone marrow and peripheral blood stem cells, or gene transfer to hematopoietic stem cells.--

Please replace the paragraph beginning on page 10, line 19, with the following rewritten paragraph:

--In the hematopoietic cells, the more mature peripheral blood cells are, the more sensitive to anticancer drugs. Utilizing this property, the present inventors attempted to concentrate hematopoietic stem cells or hematopoietic precursor cells by an anticancer drug. Namely, the colony assay was performed using spleen cells of a mouse, to which Cyclophosphamide had been administered. Results are shown in Figures 6 and 7. Figure 6 shows the number of CFR-Mix and CFU-G colonies increased 2-fold or more in the system supplemented with MK as compared with the system without MK. This experiment also indicates that MK promotes the proliferation of hematopoietic stem cells and hematopoietic precursor cells.--



Please replace the paragraph beginning on page 11, line 3, with the following rewritten paragraph:

--The effect of MK was investigated using peripheral hemocytes from a patient with non-Hodgkin's lymphoma and a MethoCult H4230 medium (consisting of methylcellulose (0.9), 2-mercaptoethanol (10 to 4 M), L-glutamine (2 mM), fetal bovine serum (30%), and bovine serum albumin (1%), and containing neither CSF nor EPO; Stem Cell Technologies Inc.). The colony assay was performed using the following combinations; MethoCult H4230 alone, H4230 + MK, H4230 + G-CSF, and H4230 + MK + G-CSF. The proportion of CD34 positive cells in peripheral stem cells from the patient was 1.4%. Results are shown in Figure 8. Although no colonies were formed with MK alone, a remarkable colony-forming capability was manifested in the case of MK + G-CSF, and the number of colonies was clearly twice or more as high as that in the case of G-CSF alone. On and after the 10<sup>th</sup> day of the initiation of assay, the increase in number of colonies tends to reduce in the MK + G-CSF group as compared with the group of G-CSF alone. Microscopic observation of colonies on and after the 10th day revealed a tendency that the colony maturation was accelerated in the MK + G-CSF group as compared with the G-CSF alone group. Therefore, the deceleration of the increase in number of colonies in the MK + G-CSF group as compared with the G-CSF alone group is probably attributed to the accelerated maturation of colonies.--

Please replace the paragraph beginning on page 11, line 23, with the following rewritten paragraph:

--It is noteworthy that, in the above-described colony assay, the size of each of colonies formed was always larger in groups supplemented with MK as compared with groups with no MK added. In order to study this fact quantitatively, three each of large colonies formed on the 14th day in the presence of MK alone, G-CSF alone, and MK + G-CSF in the colony assay of peripheral blood stem cells from the above-described patient were selected, sucked up under a microscope, and counted for their constituting cells with a hemocytometer to calculate mean values. Results are shown in Figure 9. It is obvious that colonies formed with MK + G-CSF contain more cells than those formed with G-CSF alone. That the size of colony is large means that the number of constituting cells is also large. From these results, it is evident that MK acts

on the proliferation and differentiation of hematopoietic stem cells and hematopoietic precursor cells.--

Please replace the paragraph beginning on page 12, line 10, with the following rewritten paragraph:

--The colony assay was similarly performed with the peripheral blood from a healthy normal individual. The proportion of CD34-positive cells in the peripheral blood of this subject was 0.4%. Results are shown in Figure 10. Colonies were formed with MK alone. On the 10<sup>th</sup> and 14<sup>th</sup> days, the number of colonies increased MK concentration-dependently. The MK + G-CSF system produced at the highest 2 or more times as many colonies as the system of G-CSF alone. These results clearly shows that, when MK was added, the same tendency was obtained regardless of whether cells are derived from a healthy normal individual or a patient.--

Please replace the paragraph beginning on page 12, line 19, with the following rewritten paragraph:

--Furthermore, the colony assay was similarly performed using the peripheral blood from the above-described healthy normal individual in the presence of pleiotrophin (PTN), which is another member of the MK family. PTN used herein was a recombinant PTN (pleiotrophin, recombinant human (Sf21-derived); (Lot GH055011) (R & D Systems)). Results are shown in Figure 11. PTN alone exhibited the colony-forming capability of MK and the number of colonies formed was markedly high. A synergistic action of PTN with G-CSF to promote the colony formation was similarly observed as in the case of MK. From these results, PTN obviously promotes the proliferation and differentiation of hematopoietic stem cells and hematopoietic precursor cells like MK.--

Please replace the paragraph beginning on page 13, line 3, with the following rewritten paragraph:

--Next, using a hematopoietic stem cell assay medium, complete type (Lot No. 96101601; Kyokuto Seiyaku Kogyo) containing IL-3, SCF, G-CSF, and EPO, the colony assay was carried out with peripheral blood cells from a healthy normal individual. This assay is

considered to be performed under conditions closer to *in vivo*. Results with MK are shown in Figures 12 and 13, and those with PTN in Figures 14 and 15. No increase in BFU-E was observed with any combinations including MK + IL-3, MK + SCF, and MK + G-CSF. However, the combinations of MK + EPO and PTN + EPO were assumed to increase BFU-E. Erythroblast precursor cells, BFU-E, were formed on the 14th day of culture, and are known to be more undifferentiated than CFU-E formed on the 5th to 7th days. Addition of MK or PTN to a Kyokuto complete medium resulted in formation of at the highest 2 or more times as many BFU-E as the complete medium alone on the 12th day after the initiation of culture. These results indicate that at least the addition of MK to the complete medium results in promoting the proliferation of erythroblasts as well. As described above, it is evident that the MK family is capable of acting on hematopoietic stem cells and hematopoietic precursor cells in the hematopoietic tissues of mammals to maintain, proliferate, and differentiate them, and synergistically or additionally enhancing the above-described functions by the combined use with various cytokines such as SCF, M-CSF, G-CSF, GM-CSF, IL-3 AND IL-6. Especially, the MK family remarkably promotes the proliferation of CFU-Mix, which is very close to multipotential stem cells, under conditions closer to *in vivo*. The MK family also promotes the proliferation and differentiation of granulocyte/macrophage precursor cells and exerts the remarkable neutrophil increasing effect in an *in vivo* neutropenia model. This MK family alone or in combination with more than one cytokine including SCF, M-CSF, G-CSF, GM-CSF, IL-3, and IL-6 can be clinically applied and, especially, used for the *ex vivo* expansion of hematopoietic stem cells in the transplantation of bone marrow and stem cells derived from the peripheral blood and umbilical cord blood. In addition, the MK family is expected to be used for the treatment of patients with and prevention of neutropenia, vertebrate anemia, and leukemia caused by cancer chemotherapy. Furthermore, the MK family would be used in the future for proliferating stem cells for gene therapy targeting hematopoietic stem cells. Especially, it is very promising to increase the dose density in cancer chemotherapy by the combined use of MK with G-CSF, improving effects of chemotherapy by increasing the dose of antitumor drugs or shortening the administration period.--

Please replace the paragraph beginning on page 14, line 24, with the following rewritten paragraph:

--Figure 1 illustrates the effects of single or combined use of MK, G-CSF, GM-CSF, M-CSF, IL-3, and IL-6 on the colony-forming capability of human peripheral blood mononuclear cells.--

Please replace the paragraph beginning on page 15, line 1, with the following rewritten paragraph:

--Figure 2 illustrates effects of single or combined use of MK, G-CSF, GM-CSF, M-CSF, IL-3, IL-6 and SCF on G colony, GM colony and M colony-forming capabilities of mononuclear cells in human peripheral blood different from that used in the experiment in Figure 1.--

Please replace the paragraph beginning on page 15, line 11, with the following rewritten paragraph:

--Figure 4 illustrates effects of MK on colony-forming capability of mouse spleen cells cultured in a complete methylcellulose medium containing EPO, IL-3, IL-6, and SCF (MethoCult GF M3434) supplemented with MK for 12 days in order to examine the proliferation promoting action of MK on hematopoietic cells under conditions closer to *in vivo*.--

Please replace the paragraph beginning on page 15, line 17, with the following rewritten paragraph:

--Figure 5 illustrates effects of MK on CFU-Mix colony-forming capability in an experiment similar to that in Figure 4.--

Please replace the paragraph beginning on page 15, line 19, with the following rewritten paragraph:

--Figure 6 illustrates effects of MK on CFU-G colony-forming capability when an anticancer drug, Cyclophosphamide, was administered to a mouse, and myelocytes were isolated on the 4th day after the drug administration and cultured in a complete methylcellulose medium

containing EPO, IL-3, IL-6, and SCF (MethoCult GF M3434) supplemented with MK for 14 days.--

Please replace the paragraph beginning on page 15, line 25, with the following rewritten paragraph:

--Figure 7 illustrates effects of MK on CFU-Mix colony-forming capability in an experiment similar to that in Figure 6.--

Please replace the paragraph beginning on page 15, line 27, with the following rewritten paragraph:

--Figure 8 illustrates effects of MK on colony-forming capability when peripheral blood from a patient with non-Hodgkin's lymphoma was cultured in a methylcellulose medium for the colony assay (MethoCult GF H4230) supplemented with MK, G-CSF, or MK + G-CSF for 14 days.--

Please replace the paragraph beginning on page 16, line 5, with the following rewritten paragraph:

--Figure 9 illustrates the number of colony-constituting cells on the 14<sup>th</sup> day in an experiment similar to that in Figure 8 where medium alone and that supplemented with G-CSF or MK + CSF was used.--

Please replace the paragraph beginning on page 16, line 8, with the following rewritten paragraph:

--Figure 10 illustrates effects of MK and G-CSF on colony-forming capability when peripheral blood from a healthy normal individual was cultured in a methylcellulose medium for the colony assay (MethoCult GF H4230) supplemented with MK, G-CSF, or MK + G-CSF.--

Please replace the paragraph beginning on page 16, line 14, with the following rewritten paragraph:

--Figure 12 illustrates effects of MK on CFU-E colony-forming capability when the same human peripheral blood used in the experiment of Figure 10 was cultured in a medium for the blood stem cell assay containing EPO, IL-3, G-CSF, and SCF supplemented with MK.--

Please replace the paragraph beginning on page 16, line 21, with the following rewritten paragraph:

--Figure 14 illustrates effects of PTN on CFU-E colony-forming capability when peripheral blood from the same healthy normal individual used in the experiment of Figure 10 was cultured in the same medium used in the experiment of Figure 10 supplemented with PTN in place of MK.--

Please replace the paragraph beginning on page 16, line 26, with the following rewritten paragraph:

--Figure 15 illustrates effects of PTN on BFU-E colony-forming capability in an experiment similar to that in Figure 14.--

Please replace the paragraph beginning on page 17, line 2, with the following rewritten paragraph:

--The present invention will be described below with reference to examples, but is not to be construed to be limited thereto. MK used here is human MK described in SEQ ID NO: 3 in Japanese Patent Application No. Hei 7-255354.--

Please replace the paragraph beginning on page 17, line 6, with the following rewritten paragraph:

--Example 1. Effect of MK on promoting neutrophil recovery in a neutropenia model.--

Please replace the paragraph beginning on page 17, line 8, with the following rewritten paragraph:

--Neutropenia is a disease wherein neutrophils that play the most important role in preventing infection are selectively lost or significantly reduced in number. In the following is

presented an example, wherein MK was administered in a neutropenia model prepared by administering an antitumor drug to normal mice and examined for its effect on promoting neutrophil recovery.--

Please replace the paragraph beginning on page 17, line 14, with the following rewritten paragraph:

--Neutropenia model mice were prepared by administering an antitumor drug, Cyclophosphamide (CY) to 12-week-old ICR mice (male). The mice were divided into the following groups so that each group had five mice; (1) untreated group (control group), (2) CY-administered group, (3) CY + MK-administered group, and (4) MK alone administered group. MK was diluted with physiological saline and intraperitoneally administered to the mice daily at a dose of 0.1 ml/animal and 300 µg/kg for 13 consecutive days. Six hours after the administration on the 5th day of the consecutive administration, CY was administered to the mice at a dose of 266 mg/kg, corresponding to 2/3 of the LD<sub>50</sub> value. The day of CY administration was taken as Day 0, and the blood was collected five times in total, namely on Day 0, Day 2, Day 4, Day 7, and Day 9, and counted for leukocytes, neutrophils, lymphocytes, and erythrocytes. Results are shown in Table 1. The number of lymphocytes reached the lowest value on Day 2 in the CY-administered group and the CY + MK-administered group as compared with the control group, and did not recover until Day 9. The number of neutrophils reached the lowest value on Day 4, but elevated to 2.8 times as high as that of the control group on Day 7.--

Please replace the paragraph beginning on page 18, line 9, with the following rewritten paragraph:

--For collecting human peripheral blood stem cells (PBSC), it is necessary to let them migrate from the bone marrow to the peripheral blood. A hematopoietic factor, G-CSF, was administered to an individual, who was in a hematologically stable condition, to induce migration of PBSC to the peripheral blood, and the blood was collected with a heparinized syringe. The peripheral blood was fractionated using a separation agent. The mononuclear cell layer thus fractionated was mixed with phosphate buffer (PBS), and centrifuged at 4°C and 1500

rpm for 5 min. After the centrifugation, the supernatant was discarded and cells were washed by repeating this procedure several times. The cells were suspended in a medium containing 10% FBS and counted with a hemocytometer K-8000. Finally, the cell concentration was adjusted to  $1 \times 10^6/\text{ml}$  with a medium containing 10% FBS.--

Please replace the paragraph beginning on page 18, line 22, with the following rewritten paragraph:

--Test substances used were MK, 50  $\mu\text{g}/\text{ml}$ ; G-CSF, 10  $\text{ng}/\text{ml}$ ; GM-CSF, 10  $\text{ng}/\text{ml}$ ; M-CSF, 50  $\text{ng}/\text{ml}$ ; IL-3, 10  $\text{ng}/\text{ml}$ ; IL-6, 100  $\text{ng}/\text{ml}$ ; and SCF, 10  $\text{ng}/\text{ml}$ . These substances were adjusted to the above concentrations by preparing each solution at 10-fold concentration of the final concentration using Iscove's Modified Dulbecco's Medium (IMDM) and adding it to an assay system in an amount of 10% of the total volume of the system.--

Please replace the paragraph beginning on page 19, line 22, with the following rewritten paragraph:

--Human peripheral blood mononuclear cells and test substances were prepared as in Example 2. The test substances were respectively added to the mixture containing  $1.5 \times 10^5/\text{ml}$  of cells, 30% of FBS, and 10% of the medium containing 10% BSA to give the above-described concentrations. The resulting mixture was distributed onto a plastic culture dish (Falcon, 1008) and cultured in a 5% carbon dioxide incubator at 100% humidity and 37°C for 2 weeks.--

Please replace the paragraph beginning on page 20, line 2, with the following rewritten paragraph:

--After 2 weeks, all cells were recovered from each culture dish. Cells adhering to the culture dish were recovered by treating them with 0.25 trypsin/EDTA. Cells were recovered in tubes and centrifuged once at 4°C and 800 rpm. The cells were suspended in the same volume of the culture medium and counted with a hemocytometer.--

Please replace the paragraph beginning on page 20, line 7, with the following rewritten paragraph:



--The cells collected for counting were mounted onto a slide glass with a Cytospin (Cytospin 2; SHANDON) and subjected to the esterase double staining (an esterase staining kit and esterase AS-D staining kit, Muto Kagaku Yakuin). Changing the field of vision, cells were distinguished as granulocytes, monocytes, macrophages, and others according to the staining. Results are shown in Figure 3.--

Please replace the paragraph beginning on page 20, line 15, with the following rewritten paragraph:

--The spleen of 8-week-old BDF1 mouse (female) was aseptically excised on a clean bench, and cells were pressed out using a needle (Terumo, 22G x 1 1/4" (0.70 x 32 mm)) into IMDM (GIBCO BRL) in a petri dish. Cells in the spleen were collected into IMDM (10 ml) in a tube, thoroughly mixed by pipetting, and passed through a cell strainer (FALCON 2350, 70  $\mu$ m). Mononuclear cells were counted with a hemocytometer, adjusted to a concentration of  $1 \times 10^6$  cells/ml with IMDM to serve as a cell suspension. Test solutions containing MK at 100 ng/ml, 1 mg/ml, and 10 mg/ml in IMDM were similarly prepared.--

Please replace the paragraph beginning on page 20, line 24, with the following rewritten paragraph:

--The methylcellulose medium used was a complete medium, MethoCult GF M3434 (containing 0.9% methylcellulose, 10 to 4 M 2-mercaptoethanol, 2 mM L-glutamine, FBS, 1% BSA, EPO, insulin, transferin, IL-3, IL-6 and SCF; Stem Cell Technologies, Inc.). To the medium were added the above-described cells to  $1 \times 10^5$ /ml and MK to a final concentration of 1, 10, or 100 ng/ml. The colony assay was then performed by a method similar to that in Example 2. Results are shown in Figures 4 and 5.--

Please replace the paragraph beginning on page 21, line 7, with the following rewritten paragraph:

--To concentrate hematopoietic precursor cells in myelocytes, Endoxan powder (a powerful drug according to the Japanese Pharmacopoeia, Cyclophosphamide (CY)) was administered to five 8-week-old BDF1 mice (female) at a dose of 1 mg/animal. Four days after

the administration, cells were prepared from the mouse bone marrow by the usual method, and adjusted to the concentration of  $2 \times 10^4$  cells/ml with IMDM. The colony assay was then performed in the same manner as in Example 4. Results are shown in Figures 6 and 7.--

Please replace the paragraph beginning on page 21, line 27, with the following rewritten paragraph:

--Example 8. Effects of MK and PTN on erythroblast colony formation of peripheral blood mononuclear cells from healthy normal individual.--

In the claims:

Cancel claims 1-20.

Add new claims 21-30.

After entry of the instant amendment, the claims pending in this application will read as follows.

21. A method for promoting *ex vivo* expansion of hematopoietic stem cells or hematopoietic progenitor cells, said method comprising culturing hematopoietic stem cells or hematopoietic progenitor cells with a composition comprising: (a) purified midkine (MK) protein or purified pleiotrophin (PTN) protein; and (b) one or more other purified hematopoietic growth factors.

22. A method for promoting *ex vivo* expansion of hematopoietic stem cells or hematopoietic progenitor cells, said method comprising culturing hematopoietic stem cells or hematopoietic progenitor cells with a composition comprising: (a) purified midkine (MK) protein; and (b) one or more other purified hematopoietic growth factors.

23. The method of claim 21, wherein the hematopoietic stem cells or hematopoietic progenitor cells are obtained from bone marrow, peripheral blood, or umbilical cord blood.

24. The method of claim 21, wherein the hematopoietic stem cells are colony-forming units-mix (CFU-mix).

25. The method of claim 21, wherein the one or more other purified hematopoietic growth factors are selected from the group consisting of interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte colony stimulating-factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and erythropoietin (EPO).

26. The method of claim 25, wherein the other purified hematopoietic growth factors are IL-3, IL-6, SCF, and EPO.

27. The method of claim 22, wherein the hematopoietic stem cells or hematopoietic progenitor cells are obtained from bone marrow, peripheral blood, or umbilical cord blood.

28. The method of claim 22, wherein the hematopoietic stem cells are colony-forming units-mix (CFU-mix).

29. The method of claim 22, wherein the one or more other purified hematopoietic growth factors are selected from the group consisting of interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte colony stimulating-factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and erythropoietin (EPO).

30. The method of claim 29, wherein the other purified hematopoietic growth factors are IL-3, IL-6, SCF, and EPO.

REMARKS

After entry of the instant amendment, claims 21-30 will be pending in this application, claims 1-20 having been cancelled and claims 21-30 added by the above amendment. Claims 21-30 are supported by the specification, e.g., at page 22, lines 8-26.

The title of the application has been amended as requested in an Office Action mailed March 16, 2000, in the parent application (U.S. application serial no. 09/214,569).

The specification has been amended to state the claim for priority of the instant application.

No new matter has been added by any of the above amendments.

Attached is a marked-up version of the changes being made by the current amendment.

Applicants request that all claims be examined.

Please apply any charges or credits to Deposit Account No. 06-1050.

Respectfully submitted,

Date: 2/8/02

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**Version with markings to show changes made**

In the specification:

On page 1, delete the title ("NOVEL USE OF MK FAMILY AS HEMATOPOIETIC FACTOR") and insert in its place --"USE OF THE MK FAMILY AS HEMATOPOIETIC FACTORS".--

On page 1, after the title of the application, insert

--Cross Reference to Related Applications

This application is a divisional application of U.S. Application Serial No. 09/214,569, which is a National Phase Application, filed January 7, 1999, under 35 U.S.C §371, of International Application No. PCT/JP97/02401, filed July 10, 1997.--

Paragraph beginning on page 1, line 4, has been amended as follows:

This invention relates to a novel use of MK to promote proliferation and differentiation of hematopoietic stem cells and hematopoietic precursor cells in hematopoietic tissues, peripheral blood, or umbilical cord blood synergistically with other hematopoietic factors.

Paragraph beginning on page 1, line 9, has been amended as follows:

In blood, there exist various hemocytes having different shapes and functions, including erythrocytes, leukocytes, and platelets, which play important roles in maintaining homeostasis of the living body. These mature[d] hemocytes have their own life-spans. For maintaining the hemocyte count at [the] a constant level, hemocytes must be incessantly produced to make up for the number of hemocytes that is lost due to the expiration of their life-spans.

Paragraph beginning on page 1, lines 16, has been amended as follows:

In the normal healthy individual, it is presumed that daily production of hemocytes reaches as much as  $2 \times 10^{11}$  erythrocytes,  $10^{11}$  leukocytes, and 1 to  $2 \times 10^{11}$  platelets. Hematopoietic stem cells play central rolls in the system to produce such an enormous number of

hemocytes over a long period without being exhausted. The cells have not only self-renewal capability but also multipotentiality to differentiate to various mature hemocytes including erythrocytes, granulocytes, platelets, and lymphocytes. Hematopoietic stem cells (multipotential stem cells) lose their self-renewal capability as they proliferate to become hematopoietic precursor cells (committed stem cells) destined to differentiate to the specific hemocytes. Hematopoietic precursor cells then differentiate to mature[d] peripheral hemocytes.

Paragraph beginning on page 2, line 5, has been amended as follows:

It has been known that a number of cytokines regulate each step of the hematopoietic system to proliferate and differentiate hematopoietic stem cells to various mature hemocytes via hematopoietic precursor cells. At least twenty kinds of the cytokines participating in the hematopoietic system have been found at present (Masami Bessho: Igaku no Ayumi 180(13): 802-806, 1997). [Their genes have been all cloned] The genes for all have been cloned, allowing their production on a large scale by genetic engineering techniques. Stem cell factor (SCF) and flk-2 ligand are the most remarkable cytokines as factors acting on mainly hematopoietic stem cells at the early stage of hematopoiesis. SCF acts on the most undifferentiated hematopoietic stem cells. In either mice or humans, it remarkably promotes the formation of colonies of blast colony-forming unit (CFU-BL), colony-forming unit-mixed (CFU-Mix), burst forming unit-erythrocyte (BFU-e), colony-forming unit-granulocyte/[macro-phage] macrophage (CFU-GM), eosinophil colony-forming unit (CFU-Eo), and colony-forming unit-megakaryocyte (CFU-Meg); showing a synergistic effect with various cytokines such as IL-1, IL-3, IL-4, IL-5, IL-6, IL-7, IL-11, G-CSF, GM-CSF, and EPO. It has been reported that SCF alone has [a] weak colony-stimulating activity (Tsuji, K. et al., Blood 78: 1223, 1991; Shioharu, M. et al., Blood 81: 1453, 1993; Kubo, T. and Nakahata, T., Int. Hematol. 58: 153, 1993). Nevertheless, SCF is thought to be the most important cytokine for *in vitro* amplification of hematopoietic stem cells at present.--

Paragraph beginning on page 3, line 6, has been amended as follows:

Some of these hematopoietic factors have been clinically applied. For example, erythropoietin (EPO), which promotes [to produce] the production of erythrocytes, is used for treating renal anemia, and granulocyte colony-stimulating factor (G-CSF), which promotes [to

produce] the production of neutrophils is used for treating neutropenia caused by [the] cancer chemotherapy. These contribute to improved quality of life of patients. Recently, the clinical application of thrombopoietin (TPO) for treating thrombocytopenia has been studied because it promotes the production of platelets.

Paragraph beginning on page 3, line 15, has been amended as follows:

On the one hand, since hematopoietic stem cells are capable of reconstituting all kinds of cells in the hematopoietic system, the transplantation of hematopoietic stem cells has been widely performed for hematopoietic tumors. Recently, the transplantation of peripheral blood stem cells has [become rapidly] rapidly become prevalent, and gathered attention as the powerful fundamental therapy for the chemotherapy-sensitive malignant tumors including the hematopoietic organ tumors. Furthermore, as a future prospect, the transplantation of hematopoietic stem cells is expected to be introduced to [many of the cell therapy and gene therapy] many cell therapy and gene therapy protocols. For that purpose, it is necessary to establish a method for amplifying hematopoietic stem cells *in vitro*. However, even now, human hematopoietic stem cells have been neither isolated nor clarified as to what extent they can repeat self-renewal.

Paragraph beginning on page 4, line 23, has been amended as follows:

MK was isolated as the product of a gene that is expressed at the early stage of the differentiation of mouse embryonic tumor cells by the induction of retinoic acid (Kadomatsu, K. et al., Biochem. Biophys. Res. Commun. 115: 1312-1318, 1988). PTN was found as a heparin-binding protein with [the] neurite outgrowth capability in the newborn rat brain (Rauva, H., EMBO J. 8: 2933-2941, 1989). MK and PTN belong to a new class of [heparin-binding] heparin-binding growth factors, sharing a 45% homology (in amino acid sequence) to each other, and called [as] the MK family. MK and PTN respectively exhibit characteristic expression patterns in the developmental process, indicating that they have important physiological activities for the implementation of differentiation.

Paragraph beginning on page 5, line 8, has been amended as follows:

Paying attention to such biological activities of the MK family, the present inventors studied their hematopoietic factor activities to proliferate and differentiate myeloid cells and peripheral blood stem cells of mammals. In general, [in] at what stage of the proliferation/differentiation process of hematopoietic stem cells and hematopoietic precursor cells in myeloid hematopoietic factors participate and function can be studied by culturing a certain number of myelocytes in a semi-solid medium in the presence of these hematopoietic factors, selecting cells constituting colonies formed, and counting the number of colonies. In such colony formation methods, it has been proved by a number of direct or indirect methods that, a single hematopoietic precursor cell proliferates, divides, and matures, forming a single colony comprising many matured hemocytes. There are colony formation assay methods specific for cells of each hematopoietic system including granulocyte/macrophage, erythroblasts, and megakaryocytes, and stimulators specific for each hematopoietic system are used.

Paragraph beginning on page 5, line 25, has been amended as follows:

Precursor cells of the granulocyte/macrophage system, CFU-GM, differentiate to precursor cells of neutrophil system, CFU-G, and precursor cells of monocytes, CFU-M. For that purpose, colony-simulating factors (CSF) specific to each precursor cell must be present. More specifically, [GM-CFS] GM-CSF is required for CFU-GM, G-CSF for CFU-G, and M-CSF for CFU-M. Some of these CSFs not only differentiate precursor cells to mature cells but also activate the function of matured hemocytes.

Paragraph beginning on page 6, line 6, has been amended as follows:

CFU-GM can be cultivated by either the soft agar method or the methylcellulose method using bone marrow nucleated cells. Since colonies formed by either method are constituted by a cell population of granulocytes and macrophages at various developmental stages, precursor cells one step further differentiated from hematopoietic stem cells are to be examined. Picking up and staining of these colonies revealed the presence of G colony consisting of granulocytes, M colony consisting of macrophages, and GM colony consisting of the mixture of both. In humans, colonies are rather small and classified into a group called a colony containing 40 cells or more and a group called a cluster with a lower accumulation of cells than a colony.



Paragraph beginning on page 6, lines 17, has been amended as follows:

In order to examine whether MK has the activity [to proliferate myelocytes] causing myelocytes to proliferate, the proliferation of mouse myelocytes was assayed by the MTT method, resulting in enhancing the proliferation 1.6- to 2-fold in the system supplemented with MK at the concentrations of 5, 50, 500, and 5000 ng/ml as compared with the system without MK. A concentration-dependent elevation of the activity was observed in the range of 5 to 500 ng/ml MK.

Paragraph beginning on page 6, line 24, has been amended as follows:

In the colony assay for human peripheral blood mononuclear cells in the presence of various cytokines, as shown in Figure 1, colonies were not formed at all in the system without cytokines, but formed in the system supplemented with MK similarly as with GM-CSF and IL-3. The colony size tends to be larger in the system [added] with MK added than in the systems [added] with cytokines such as G-CSF, GM-CSF, and IL-3 added. These results indicate that MK alone has the activity to maintain the viability of human peripheral blood stem cells or hematopoietic precursor cells, or promote their proliferation. Furthermore, the combined use of MK with other cytokines such as M-CSF, G-CSF, GM-CSF, IL-3, and IL-6 synergistically promotes [the] colony-forming capability. For example, the number of colonies increased 7 to 9-fold in the cases of combined use of MK with G-CSF, GM-CSF, or IL-3 as compared with those of the single use of MK, G-CSF, GM-CSF, or IL-3. Also, the combination of MK + G-CSF + IL-3 + IL-6 remarkably increased the number of colonies formed as compared with that of GM-CSF + IL-3 + IL-6, and the combination of MK + G-CSF + IL-6 significantly increased the number of colonies as compared with that of G-CSF + IL-6. [Similarly, when the colony assay was performed with human peripheral blood mononuclear cells different from those used in Figure - 1 in the presence of various cytokines and cells constituting colonies were morphologically observed as shown in Figure 2, GM colony was formed with the single use of MK, GM-CFS, or IL-3, and G colony was mainly formed with G-CSF alone] In another experiment using a source of human peripheral blood mononuclear cells different from that used for the experiments described in Fig. 1, treatment with MK, GM-CSF, or IL-3 alone produced primarily GM colonies, while use of G-CSF alone produced primarily G colonies. Combinations

of MK +G-CSF + GM-CSF, or [that] of MK + G-CSF significantly increased the number of G [colony] colonies as compared with the [single] use of G-CSF alone. That is, MK is considered to synergistically promote the proliferation, differentiation and maturation of CFU-GM of G-CSF, increasing the number of neutrophils in the peripheral blood.

Paragraph beginning on page 7, line 25, has been amended as follows:

When cells after 2-week liquid culture of human peripheral blood stem cells in the presence of various cytokines were examined by specific staining, there were observed, as shown in Figure 3, predominantly many granulocytes (neutrophils) in the system supplemented with MK, clearly indicating the action of MK on the proliferation of neutrophils. Especially, in the case of the combination of MK + G-CSF +GM-CSF + SCF + IL-3 + IL-6, there were observed an extremely remarkable promotion of the proliferation and differentiation of neutrophils.

Paragraph beginning on page 8, line 6, has been amended as follows:

Also, in the colony assay performed after the above-described liquid culture, the cell adherence to a culture dish increased in the system supplemented with MK as compared with that without MK, indicating that MK also promotes the proliferation of the interstitial cell system (stroma cell system). In the case of IL-6 alone, colonies formed were of macrophages, and in the case of MK + IL-6, half of colonies formed were [those] of granulocytes. These results indicate the participation of MK in the promotion of proliferation and differentiation of granulocytes.

Paragraph beginning on page 8, line 15, has been amended as follows:

Whether such a remarkable promotion by MK of the production of neutrophils is displayed *in vivo* can be studied by administering MK to a mouse whose hematopoietic system has been damaged by administration of an anticancer drug, or exposure to radiation, and examining the recovery state of neutrophils. MK was administered to a mouse daily for [consecutive] 13 consecutive days, and on the 5th day after the initiation of administration, an anticancer drug, Cyclophosphamide (CY), was administered to the mouse. Examination of hemocytes in the blood collected from the mouse at appropriate intervals revealed a remarkable promotion of the recovery of the number of neutrophils as expected (Table 1).

Paragraph spanning pages 9 and 10 has been amended as follows:

Results of the colony assay for mouse spleen cells in the system of the M3434 medium supplemented with MK are shown in Figures 4 and 5. Figure 4 illustrates the number of CFU-GM [colony] colonies or CFU-G [colony] colonies. Although CFU-GM [colony] colonies or CFU-G [colony] colonies can be formed with the M3434 medium alone, the number of colonies generally increases in the system supplemented with the MK as compared with the M3434 medium alone. Especially, when MK is added at the concentration of 1 to 10 ng/ml, the colony number remarkably increased 2 to 3-fold on the 8th and 10th day of the assay. Figure 5 illustrates the number of colony-forming unit-mixed (CFU-Mix). CFU-Mix are multipotential stem cells at a slightly differentiated stage, having lower self-renewal capability than blast colony-forming units (CFU-BL) which are the most undifferentiated cells identifiable by the *in vitro* colony assay and are thought to contain cells capable of differentiating to erythrocytes, leukocytes, and platelets. In the system supplemented with MK, CFU-Mix colony significantly increased in number. That is, MK, at least by its combined use with IL-3, IL-6, SCF and EPO, is thought to significantly promote the proliferation and differentiation of hematopoietic stem cells or immature hematopoietic cells close to them. These activities are thought to be very useful for the proliferation of hematopoietic stem cells *in vitro* for the transplantation of bone marrow and peripheral blood stem cells, or gene transfer to hematopoietic stem cells.

Paragraph beginning on page 10, line 19, has been amended as follows:

In the hematopoietic cells, the more mature[d] peripheral blood cells are, the more sensitive to anticancer drugs. Utilizing this property, the present inventors attempted to concentrate hematopoietic stem cells or hematopoietic precursor cells by an anticancer drug. Namely, the colony assay was performed using spleen cells of a mouse, [which had been administered with Cyclophosphamide] to which Cyclophosphamide had been administered. Results are shown in Figures 6 and 7. Figure 6 shows the number of CFU-Mix and CFU-G colonies increased [2-told] 2-fold or more in the system supplemented with MK as compared with the system without MK. This experiment also indicates that MK promotes the proliferation of hematopoietic stem cells and hematopoietic precursor cells.

Paragraph beginning on page 11, line 3, has been amended as follows:

[Effect] The effect of MK was investigated using peripheral hemocytes from a patient with non-Hodgkin's lymphoma and a MethoCult H4230 medium [ ] (consisting of methylcellulose (0.9%), 2-mercaptoethanol (10 to 4 M), L-glutamine (2 mM), fetal bovine serum (30%), and bovine serum albumin (1%), and containing neither CSF nor EP0; StemCell Technologies Inc.[ ]). [Colony] The colony assay was performed using the following combinations; MethoCult H4230 alone, H4230 + MK, H4230 + G-CSF, and H4230 + MK + G-CSF. The [ratio] proportion of CD34 positive cells in peripheral stem cells from the patient was 1.4%. Results are shown in Figure 8. Although no colonies were formed [at all] with MK alone, a remarkable colony-forming capability was manifested in the case of MK + G-CSF, and the number of colonies was clearly twice or more as high as that in the case of G-CSF alone. On and after the 10th day of the initiation of assay, the increase in number of colonies tends to reduce in the MK + G-CSF group as compared with the group of G-CSF alone. Microscopic observation of colonies on and after the 10th day revealed a tendency that the colony maturation was accelerated in the MK + G-CSF group as compared with the G-CSF alone group. Therefore, the deceleration of the increase in number of colonies in the MK + G-CSF group as compared with the G-CSF alone group is probably attributed to the accelerated maturation of colonies.

Paragraph beginning on page 11, line 23, has been amended as follows:

It is noteworthy that, in the above-described colony assay, the size of each of colonies formed was always larger in groups supplemented with MK as compared with groups [added] with no MK added. In order to study this fact quantitatively, three each of large colonies formed on the 14th day in the presence of MK alone, G-CSF alone, and MK + G-CSF in the colony assay of peripheral blood stem cells from the above-described patient were selected, sucked up under a microscope, and counted for their constituting cells with a hemocytometer to calculate mean values. Results are shown in Figure 9. It is obvious that colonies formed with MK+ G-CSF contain more [constituting] cells than those formed with G-CSF alone. That the size of colony is large means that the number of constituting cells is also large. From these results, it is

evident that MK acts on the proliferation and differentiation of hematopoietic stem cells and hematopoietic precursor cells.

Paragraph beginning on page 12, line 10, has been amended as follows:

The colony assay was similarly performed with the peripheral blood from a healthy normal individual. The [ratio] proportion of CD34-positive cells in the peripheral blood of this subject was 0.4%. Results are shown in Figure 10. Colonies were formed with MK alone. On the 10th and 14th days, the number of colonies increased MK concentration-dependently. The MK + [G-CSG] G-CSF system produced at the highest [twice or more] 2 or more times as many colonies as the system of G-CSF alone. These results clearly shows that, when MK was added, the same tendency was obtained regardless of whether cells are derived from a healthy normal individual or a patient.

Paragraph beginning on page 12, line 19, has been amended as follows:

Furthermore, the colony assay was similarly performed using the peripheral blood from the above-described healthy normal individual in the presence of pleiotrophin (PTN), which is another member of the MK family. PTN used herein was a recombinant PTN [ ](pleiotrophin, recombinant human (Sf21-derived); (Lot GH055011) (R &D Systems)[ ]). Results are shown in Figure 11. PTN alone exhibited the colony-forming capability [like] of MK and the number of colonies formed was markedly high. A synergistic action of PTN with G-CSF to promote the colony formation was similarly observed as in the case of MK. From these results, PTN obviously promotes the proliferation and differentiation of hematopoietic stem cells and hematopoietic precursor cells like MK.

Paragraph beginning on page 13, line 3, has been amended as follows:

Next, using a hematopoietic stem cell assay medium, complete type (Lot No. 96101601; Kyokuto Seiyaku Kogyo) containing IL-3, SCF, G-CSF, and EPO, the colony assay was carried out with peripheral blood cells from a healthy normal individual. This assay is considered to be performed under conditions closer to *in vivo*. Results with MK are shown in Figures 12 and 13, and those with PTN in Figures 14 and 15. No increase in BFU-E was observed with any

combinations including MK+ IL-3, MK + SCF, and MK + G-CSF. However, the combinations of MK + EPO and PTN + EPO were assumed to increase BFU-E. Erythroblast precursor cells, BFU-E, were formed on the 14th day of culture, and are known to be more undifferentiated than CFU-E formed on the 5th to 7th days. Addition of MK or PTN to a Kyokuto complete medium resulted in formation of at the highest [twice or more] 2 or more times as many BFU-E as the complete medium alone on the 12th day after the initiation of culture. These results indicate that at least the addition of MK to the complete medium results in promoting the proliferation of erythroblasts as well. As described above, it is evident that the MK family is capable of acting on hematopoietic stem cells and hematopoietic precursor cells in the hematopoietic tissues of mammals to maintain, proliferate, and differentiate them, and synergistically or additionally enhancing the above-described functions by the combined use with various cytokines such as SCF, M-CSF, G-CSF, GM-CSF, IL-3 and IL-6. Especially, the MK , family remarkably promotes the proliferation of CFU-Mix, which is very close to multipotential stem cells, under conditions closer to *in vivo*. The MK family also promotes the proliferation and differentiation of granulocyte/macrophage precursor cells and exerts the remarkable neutrophil increasing effect in an *in vivo* neutropenia model. This MK family alone or in combination with more than one [kinds of cytokines] cytokine including SCF, M-CSF, G-CSF, GM-CSF, IL-3, and IL-6 can be clinically applied and, especially, used for the *ex vivo* expansion of hematopoietic stem cells in the transplantation of bone marrow and stem cells derived from the peripheral blood and umbilical cord blood. In addition, the MK family is expected to be used for the treatment of patients with and prevention of [patient with] neutropenia, vertebrate anemia, and leukemia caused by cancer chemotherapy. Furthermore, the MK family would be used in the future for proliferating stem cells for gene therapy targeting hematopoietic stem cells [in future]. Especially, it is very promising to increase the dose density in cancer chemotherapy by the combined use of MK with G-CSF, improving effects of chemotherapy by increasing the dose of antitumor drugs or shortening the administration period.

Paragraph beginning on page 14, line 24, has been amended as follows:

Figure 1 illustrates the effects of [the] single or combined use of MK, G-CSF, GM-CSF, M-CSF, IL-3, and IL-6 on the colony-forming capability of human peripheral blood mononuclear cells.

Paragraph beginning on page 15, line 1, has been amended as follows:

Figure 2 illustrates effects of [the] single or combined use of MK, G-CSF, GM-CSF, M-CSF, IL-3, IL-6 and SCF on G colony, GM colony and M colony-forming capabilities of mononuclear cells in [the] human peripheral blood different from that used in the experiment in Figure 1.

Paragraph beginning on page 15, line 11, has been amended as follows:

Figure 4 illustrates effects of MK on [the] colony-forming capability of mouse spleen cells cultured in a complete methylcellulose medium containing EPO, IL-3, IL-6, and SCF (MethoCult GF M3434) supplemented with MK for 12 days in order to examine the proliferation promoting action of MK on hematopoietic cells under conditions closer to *in vivo*.

Paragraph beginning on page 15, line 17, has been amended as follows:

Figure 5 illustrates effects of MK on [the] CFU-Mix colony-forming capability in an experiment similar to that in Figure 4.

Paragraph beginning on page 15, line 19, has been amended as follows:

Figure 6 illustrates effects of MK on [the] CFU-G colony-forming capability when an anticancer drug, Cyclophosphamide, was administered to a mouse, and myelocytes were isolated on the 4<sup>th</sup> day after the drug administration and cultured in a complete methylcellulose medium containing EPO, IL-3, IL-6, and SCF (MethoCult GF M3434) supplemented with MK for 14 days.

Paragraph beginning on page 15, line 25, has been amended as follows:

Figure 7 illustrates effects of MK on [the] CFU-Mix colony-forming capability in an experiment similar to that in Figure 6.

Paragraph beginning on page 15, line 27, has been amended as follows:

Figure 8 illustrates effects of MK on [the] colony-forming capability when [the] peripheral blood from a patient with non-Hodgkin's lymphoma [were] was cultured in a methylcellulose medium for the colony assay (MethoCult GF H4230) supplemented with MK, G-CSF, or MK + G-CSF for 14 days.

Paragraph beginning on page 16, line 5, has been amended as follows:

Figure 9 illustrates the number of colony-constituting cells on the 14th day in an experiment similar to that in Figure 8 where [the] medium alone and that supplemented with G-CSF or MK + G-CSF was used.

Paragraph beginning on page 16, line 8, has been amended as follows:

Figure 10 illustrates effects of MK and G-CSF on [the] colony-forming capability when [the] peripheral blood from a healthy normal individual was cultured in a methylcellulose medium for the colony assay (MethoCult GF H4230) supplemented with MK, G-CSF, or MK + G-CSF.

Paragraph beginning on page 16, line 14, has been amended as follows:

Figure 12 illustrates effects of MK on [the] CFU-E colony-forming capability when the same human peripheral blood used in the experiment of Figure 10 was cultured in a medium for the blood stem cell assay containing EPO, IL-3, G-CSF, and SCF supplemented with MK.

Paragraph beginning on page 16, line 21 has been amended as follows:

Figure 14 illustrates effects of PTN on [the] CFU-E colony-forming capability when [the] peripheral blood from the same healthy normal individual used in the experiment of Figure 10 was cultured in the same medium used in the experiment of Figure 10 supplemented with PTN in place of MK.

Paragraph beginning on page 16, line 26, has been amended as follows:



Figure 15 illustrates effects of PTN on [the] BFU-E colony-forming capability in an experiment similar to that in Figure 14.

Paragraph beginning on page 17, line 2, has been amended as follows:

The present invention will be described below with reference to examples, but is not to be considered to be limited thereto. MK used herein is human MK described in SEQ ID NO: 3 in Japanese Patent Application No. Hei 7-255354.

Paragraph beginning on page 17, line 6, has been amended as follows:

Example 1. Effect of MK on promoting neutrophil recovery in a [neutropenia] neutropenia model.

Paragraph beginning on page 17, line 8, has been amended as follows:

Neutropenia is a disease wherein neutrophils that play the most important role in preventing infection are selectively lost or significantly reduced in number. In the following is presented an example, wherein MK was administered [to] in a neutropenia model prepared by administering an antitumor drug to normal mice and examined for its effect on promoting neutrophil recovery.

Paragraph beginning on page 17, line 14, has been amended as follows:

Neutropenia model mice were prepared by administering an antitumor drug, Cyclophosphamide (CY) to 12-week-old ICR mice (male). The mice were divided into the following groups so that each group had five mice; (1) untreated group (control group), (2) CY-administered group, (3) CY + MK-administered group, and (4) MK alone administered group. MK was diluted with [a] physiological saline and [intrapenitoneally] intraperitoneally administered to the mice daily at a dose of 0.1 ml/animal and 300 µg/kg for 13 consecutive days. Six hours [later] after the administration on the 5th day of the consecutive administration, CY was administered to the mice at a dose of 266 mg/kg, corresponding to 2/3 of the LD<sub>50</sub> value. The day of CY administration was taken as Day 0, and the blood was collected five times in

total, namely on Day 0, Day 2, Day 4, Day 7, and Day 9, and counted for leukocytes, neutrophils, lymphocytes, and erythrocytes. Results are shown in Table 1. The number of lymphocytes reached the lowest value on Day 2 in the CY-administered group and the CY + MK-administered group as compared with the control group, and did not recover until Day 9. The number of neutrophils reached the lowest value on Day 4, but elevated to 2.8 times as high as that of the control group on Day 7.

Paragraph beginning on page 18, line 9, has been amended as follows:

For collecting human peripheral blood stem cells (PBSC), it is necessary to let them migrate from the bone marrow to the peripheral blood. A hematopoietic factor, G-CSF, was administered to an individual, who [is in the hematologically stable conditions] was in a hematologically stable condition, to induce migration of PBSC to the peripheral blood, and the blood was collected with a heparinized syringe. The peripheral blood was fractionated using a separation agent. The mononuclear cell layer thus fractionated was mixed with [a] phosphate buffer (PBS), and centrifuged at 4°C and 1500 rpm for 5 min. After the centrifugation, the supernatant was discarded and cells were washed by repeating this procedure several times. The cells were suspended in a medium containing 10% FBS and counted with a hemocytometer K-8000. Finally, the cell concentration was adjusted to  $1 \times 10^6/\text{ml}$  with a medium containing 10% FBS.

Paragraph beginning on page 18, line 22, has been amended as follows:

Test substances used were MK, 50 µg/ml; G-CSF, 10 ng/ml; GM-CSF, 10 ng/ml; M-CSF, 50 ng/ml; IL-3, 10 ng/ml; IL-6, 100 ng/ml; and SCF, 10 ng/ml. These substances were adjusted to [have] the above concentrations by preparing each solution [with] at 10-fold concentration of the final concentration using Iscove's Modified Dulbecco's Medium (IMDM) and adding it to an assay system in an amount of 10% of the total volume of the system.

Paragraph beginning on page 19, line 22, has been amended as follows:

Human peripheral blood mononuclear cells and test substances were prepared [similarly] as in Example 2. The test substances were respectively added to the mixture containing  $1.5 \times$

10<sup>5</sup>/ml of cells, 30% of FBS, and 10% of the medium containing 10% BSA to give the above-described concentrations. The resulting mixture was distributed onto a plastic culture dish (Falcon, 1008) and cultured in a 5% carbon dioxide incubator at 100% humidity and 37°C for 2 weeks.

Paragraph beginning on page 20, line 2, has been amended as follows:

After 2 weeks, all cells were recovered from each culture dish. Cells adhering to the culture dish were recovered by treating them with 0.25% trypsin/EDTA. Cells were recovered in tubes and centrifuged once at 4°C and 800 rpm. The [thus-collected] cells were suspended in the same volume of the culture medium and counted with a hemocytometer.

Paragraph beginning on page 20, line 7, has been amended as follows:

The cells collected for counting were mounted onto a slide glass with a Cytospin (Cytospin 2; SHANDON) and subjected to the esterase double staining (an esterase staining kit and esterase AS-D staining kit, Muto Kagaku Yakuhin). Changing the field of vision, cells were distinguished [into] as granulocytes, monocytes, macrophages, and others according to the [stainability] staining. Results are shown in Figure 3.

Paragraph beginning on page 20, line 15, has been amended as follows:

The spleen of 8-week-old BDF1 mouse (female) was aseptically excised [in] on a clean bench, and cells were pressed out using a needle [[[Therumo, 22G x 1 1/4" (0.70 x 32 mm)]]] into IMDM (GIBCO BRL) in a petri dish. Cells in the spleen were collected into IMDM (10 ml) in a tube, thoroughly mixed by pipetting, and passed through a cell strainer (FALCON 2350, 70  $\mu$  m). Mononuclear cells were counted with a hemocytometer, adjusted to [the concentration] a concentration of 1 x 10<sup>6</sup> cells/ml with IMDM to serve as a cell suspension. Test solutions containing MK at 100 ng/ml, 1 mg/ml, and 10 mg/ml in IMDM were similarly prepared.

Paragraph beginning on page 20, line 24, has been amended as follows:

The methylcellulose medium used was a complete medium, MethoCult GF M3434 (containing 0.9% methylcellulose, 10 to 4 M 2-mercaptoethanol, 2 mM L-glutamine, FBS, 1%

BSA, EPO, insulin, transferrin, IL-3, IL-6 and SCF; Stem Cell Technologies, Inc.). To the medium were added the above-described cells to  $1 \times 10^5/\text{ml}$  and MK to a final concentration of 1, 10, or 100 ng/ml. The colony assay was then performed by a [similar method as] method similar to that in Example 2. Results are shown in Figures 4 and 5.

Paragraph beginning on page 21, line 7, has been amended as follows:

To concentrate hematopoietic precursor cells in myelocytes, Endoxan powder [ ](a powerful drug according to the Japanese Pharmacopoeia, Cyclophosphamide (CY)[ ]) was administered to five 8-week-old BDF1 mice (female) at a dose of 1 mg/animal. Four days after the administration, cells were prepared from the mouse bone marrow by the usual method, and adjusted to the concentration of  $2 \times 10^4$  cells/ml with IMDM. The colony assay was then performed in the same manner as in Example 4. Results are shown in Figures 6 and 7.

Paragraph beginning on page 21, line 27, has been amended as follows:

Example 8. Effects of MK and PTN on [the] erythroblast colony formation of peripheral blood mononuclear cells from healthy normal individual.

In the claims:

Please cancel claims 1-20.

Please add claims 21-30:

--21. A method for promoting *ex vivo* expansion of hematopoietic stem cells or hematopoietic progenitor cells, said method comprising culturing hematopoietic stem cells or hematopoietic progenitor cells with a composition comprising: (a) purified midkine (MK) protein or purified pleiotrophin (PTN) protein; and (b) one or more other purified hematopoietic growth factors.

22. A method for promoting *ex vivo* expansion of hematopoietic stem cells or hematopoietic progenitor cells, said method comprising culturing hematopoietic stem cells or hematopoietic progenitor cells with a composition comprising: (a) purified midkine (MK) protein; and (b) one or more other purified hematopoietic growth factors.

23. The method of claim 21, wherein the hematopoietic stem cells or hematopoietic progenitor cells are obtained from bone marrow, peripheral blood, or umbilical cord blood.

24. The method of claim 21, wherein the hematopoietic stem cells are colony-forming units-mix (CFU-mix).

25. The method of claim 21, wherein the one or more other purified hematopoietic growth factors are selected from the group consisting of interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte colony stimulating-factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and erythropoietin (EPO).

26. The method of claim 25, wherein the other purified hematopoietic growth factors are IL-3, IL-6, SCF, and EPO.

27. The method of claim 22, wherein the hematopoietic stem cells or hematopoietic progenitor cells are obtained from bone marrow, peripheral blood, or umbilical cord blood.

28. The method of claim 22, wherein the hematopoietic stem cells are colony-forming units-mix (CFU-mix).

29. The method of claim 22, wherein the one or more other purified hematopoietic growth factors are selected from the group consisting of interleukin-3 (IL-3), interleukin-6 (IL-6), granulocyte colony stimulating-factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), stem cell factor (SCF), and erythropoietin (EPO).

30. The method of claim 29, wherein the other purified hematopoietic growth factors are IL-3, IL-6, SCF, and EPO.--

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